

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
30 August 2001 (30.08.2001)

PCT

(10) International Publication Number  
**WO 01/62897 A1**

(51) International Patent Classification<sup>7</sup>: C12N 5/06,  
15/10, A61K 35/12, A01K 67/027, C12N 15/63, C07K  
14/47, C12N 15/12, A61P 25/28

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(21) International Application Number: PCT/EP01/02127

(22) International Filing Date: 21 February 2001 (21.02.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:  
00200671.6 25 February 2000 (25.02.2000) EP

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(81) Designated States (national): AE, AG, AL, AM, AT, AU,  
AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ,  
DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR,  
HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR,  
LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ,  
NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM,  
TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States (regional): ARIPO patent (GH, GM,  
KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian  
patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European  
patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE,  
IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF,  
CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

**Published:**

- with international search report
- before the expiration of the time limit for amending the  
claims and to be republished in the event of receipt of  
amendments

For two-letter codes and other abbreviations, refer to the "Guid-  
ance Notes on Codes and Abbreviations" appearing at the begin-  
ning of each regular issue of the PCT Gazette.

(54) Title: PRESENILIN DEFICIENT MULTIPOTENT CELL LINES AND SCREENING METHODS FOR INTRAMEM-  
BRANE REGULATED PROTEOLYTIC ACTIVITIES USING THESE LINES

(57) Abstract: The present invention relates to the field of neurological and physiological dysfunctions associated with Alzheimer's disease. More particularly to mutant embryonic stem (ES) cell lines characterized by no detectable  $\gamma$ -secretase activity, derived from double presenilin (PS 1 and PS 2) knock-out mice embryos. These cell lines can be used for in vitro screening of molecules and products involved in regulated intramembrane proteolysis of proteins such as the PP, the APP-like proteins, Notch, Ire-1p, and other integral membrane proteins; to identify proteases responsible for the latter proteolysis, like gamma-secretases, or proteins involved in the control of these proteolytic activities. These mutant ES cell lines can be manipulated to differentiate into fibroblast, neurons, myocytes or can be used to generate novel transgenic mice. Moreover, a reporter system comprising a chimeric molecule to detect the above mentioned intramembrane proteolysis or modulators thereof.

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produced. This A $\beta$ 42-peptide forms amyloid fibrils more readily than the A $\beta$ 40-peptide, which is normally produced via wild type APP, PS1 and PS2. These insoluble, amyloid fibrils are deposited in amyloid plaques, one of the neuropathological hallmarks in the brains of patients suffering from AD. These A $\beta$ -peptides are generated from the amyloid precursor protein (APP), by distinct proteolytic activities. The  $\beta$ -secretase was recently identified and is a type I integral membrane aspartyl protease, also called BACE (Vassar *et al.*, 1999, Science 286, 735). BACE cleaves APP at the aminotermminus of the amyloid peptide sequence in APP. The elusive  $\gamma$ -secretase cuts APP at the carboxyterminus of the amyloid peptide. Although it is still unclear whether one, two or several different enzymes are involved in this process, it can be stated that the secretases are important and are molecular targets for drug discovery since it is believed that abnormal processing of APP is involved in the pathogenesis of both genetic and sporadic Alzheimer's Disease. It is also clear that the molecular identification of endogenous proteins involved either directly or indirectly in secretase activities is of uttermost importance.

Recent research has demonstrated the involvement of presenilins in the formation of amyloid- $\beta$  through their effects on  $\gamma$ -secretase(s). These findings establish a genetic link between presenilins and  $\gamma$ -secretase(s) and make them potential molecular targets for developing compounds to prevent or treat AD.

Presenilins (PS) are polytransmembrane proteins located in the endoplasmic reticulum and the early Golgi apparatus. Missense mutations cause familial Alzheimer's disease (AD) in a dominant fashion. The exact pathogenic mechanism underlying the disease process is not fully unravelled, but it is fairly established that most PS missence mutations effect the processing of the amyloid precursor protein (APP), resulting in an increased generation of the longer form of the amyloid peptide (the A $\beta$ 42-peptide) which is a major component of the amyloid plaques in patients as stated above.

The inactivation of the PS1-gene in mice results in a severe lethal phenotype, characterised by late embryonic lethality, disturbed somitogenesis, mid-line closure deficiencies and malformations of the central nervous system, most significantly underdevelopment of the subventricular zone and a neuronal migration disorder mimicking human lissencephaly type II (Hartmann *et al.*, 1999, Curr. Biol.9, 719). Cell biological studies in PS1-deficient neurons have demonstrated that PS1 deficiency interferes with the  $\gamma$ -secretase-mediated proteolysis of the transmembrane domain of APP and an estimated reduction of 85%  $\gamma$ -secretase-mediated proteolysis was



influence of the PS2-gene. In contrast to PS1 knock-out mice, PS2 knock-out mice are viable and fertile and develop only mild pulmonary fibrosis and hemorrhage with age (Herreman *et al.*, 1999, Proc.Natl.Acad.Sciences 96, 11872). Quite surprisingly and unexpectedly, the absence of PS2 does not detectably alter the processing of amyloid

precursor protein.

In a further step the complete deletion of both PS2 and PS1 genes was therefore pursued. The phenotype of these mice closely resembles the phenotype of mice that are fully deficient in Notch-1. These observations demonstrate that PS1 and PS2 have partially overlapping functions and that PS1 is essential and PS2 is redundant for normal Notch signalling during mammalian embryological development. Biochemical analysis of the exact effects of the double PS deletion on Notch signaling, APP processing, the UPR and other biological processes, is however hampered by the fact that only a limited number of cells can be obtained from such embryos. This has been circumvented by immortalizing these cells using transfection with large T or myc cDNA constructs. Although this allows to obtain large amounts of presenilin negative cells, these procedures also largely interfere with important cellular signaling mechanisms and also lead to genetic instability of the cells. It is therefore difficult to assess correctly to what extent phenotypical alterations in these cells are caused by the presenilin deficiency in se or by secondary alterations caused by the immortalisation procedure.

It is thus clear that the development of clean and genetically stable cell lines, without activity of PS1 and PS2, is needed in order to understand the above described biological pathways, and especially the role of the presenilins, their mutations and deficiencies, in the pathogenesis of AD.

The present invention provides embryonic stem (ES) cell lines generated from double presenilin (PS1 and PS2) knockout mice. Surprisingly, given the residual  $\gamma$ -secretase activity in PS1 knock out cells and the absence of effects on  $\gamma$ -secretase activity in PS2 knock out cells, it was found that  $\gamma$ -secretase activity dropped to an undetectable level in these mutant cell lines. Accordingly, the latter cell lines can be used to screen for  $\gamma$ -secretase activity and modulators thereof. The present invention also provides a reporter system to detect  $\gamma$ -secretase activity and modulators thereof.



**Figure 4:****A cell based  $\gamma$ -secretase assay**

Panel A: The APP ectodomain and transmembrane domain are fused to the intracellular domain of Notch. Luciferase cDNA is fused to the Hes-1 promotor fragment as indicated in the text. Cells are transfected with these constructs. Proteolytic release of the Notch intracellular domain by a  $\gamma$ -secretase activity induces luciferase activity as indicated, allowing to monitor efficiently  $\gamma$ -secretase activity. (CSL is present in the transfected cells).

Panel B: Different constructs are displayed. The first series are chimeric proteins containing progressively shortened Notch intracellular domain fragments fused to wild type APP (see text for details). The next construct contains the Swedish mutation of APP to increase  $\beta$ -secretase cleavage. The last construct is similar to the first construct, but the APP ectodomain was truncated at the  $\beta$ -secretase site.

Panel C: pSG5APP-NIC and pSG5APP<sup>sw</sup>-NIC constructs were transfected in Cos and Hela cells. Reaction with antibodies against the N terminus of APP (22C11), or against the cytoplasmic domain of APP or with the monoclonal 9E10 (myc tag) demonstrated in western blotting a protein with a molecular mass around 150 kDa corresponding to the predicted fusion protein (indicated as APPNIC).  $\alpha$  or  $\beta$  secretase cleaves the extracellular domain of APP producing a soluble ectodomain APPs and a membrane associated carboxy-terminal fragments (APP/NIC CT fragments). Amyloid- $\beta$  peptide and p3 fragment are also produced, as indicated in the final panel. The production of amyloid and p3 peptide is less efficient from the chimeric construct than from wild type APP. One possible reason is that the endocytosis signals in the APP cytoplasmic tail, important for amyloid production in wild type APP are not present in the chimeric protein.

Panel D: Results with different constructs are displayed. Significant induction of luciferase activity is shown after transfection with the chimeric constructs. The different APP/NIC chimeric proteins have very similar induction efficiencies.

**Aims and detailed description of the present invention**

The present invention aims at providing embryonic stem cell lines in which the residual  $\gamma$ -secretase activity is reduced by more than 90%, preferentially more than 99%, and



increase the sensitivity of such assays it can be considered to stably transfect the ES cells with cDNA's encoding APP (human, containing clinical or synthetic mutations), Notch (possibly mutated or modified), or other proteins and reporters useful for such assays.

- 5 In another embodiment of the invention the double mutant presenilin ES cell line can be used as a cellular background to express presenilin clinical mutations. Such a cell line can then be used to screen for inhibitors that specifically inhibit the production of pathogenic amyloid- $\beta$ 42-peptide. Indeed, the double mutant presenilin ES cell line transformed with a Alzheimer's disease causing PS1 mutation is predicted to produce
- 10 predominantly the amyloid- $\beta$ 42 form whereas the mutant ES cell line transformed with the wild type PS1 is predicted to produce mostly the non-pathogenic amyloid- $\beta$ 40 peptide. These cell lines can thus be used in differential drug screening approaches to identify compounds which inhibit preferentially the amyloid- $\beta$ 42 formation and not the amyloid- $\beta$ 40 peptide generation. By comparing the differential effect of compounds on
- 15 the amyloid peptide production in the mutant cell lines overexpressing wild type presenilin and clinical mutant presenilin, compounds affecting the pathological amyloid peptide (amyloid- $\beta$ 42 peptide) production can specifically be detected. A compound able to interfere with the formation of amyloid- $\beta$ 42 peptide and not with the formation of amyloid- $\beta$ 40 peptide should at least have a 20% reduced amyloid- $\beta$ 42 peptide
- 20 formation, preferentially at least a 50% reduced amyloid- $\beta$ 42 peptide formation and more preferentially at least a 90% reduced amyloid- $\beta$ 42 peptide formation.

- In another embodiment the invention provides a method for the production of a pharmaceutical composition comprising the usage of an embryonic stem cell line to identify a gene coding for a protein having gamma-secretase activity or a compound
- 25 that specifically interferes with the formation of the A $\beta$ 42 – peptide and not with the formation of the A $\beta$ 40 – peptide, and further more mixing the gene or compound identified or a derivative or homologue thereof with a pharmaceutically acceptable carrier. The administration of a gene or compound or a pharmaceutically acceptable salt thereof may be by way of oral, inhaled or parenteral administration. The active
- 30 compound may be administered alone or preferably formulated as a pharmaceutical composition. A unit dose will normally contain 0.01 to 50 mg for example 0.01 to 10 mg, or 0.05 to 2 mg of compound or a pharmaceutically acceptable salt thereof. Unit doses will normally be administered once or more than once a day, for example 2,



aerosol or solution for a nebulizer, or as a microfine powder for insufflation, alone or in combination with an inert carrier such as lactose. In such a case the particles of active compound suitably have diameters of less than 50 microns, preferably less than 10 microns, for example between 1 and 5 microns, such as between 2 and 5 microns. A  
5 favored inhaled dose will be in the range of 0.05 to 2 mg, for example 0.05 to 0.5 mg, 0.1 to 1 mg or 0.5 to 2 mg. For parenteral administration, fluid unit dose forms are prepared containing a compound of the present invention and a sterile vehicle. The active compound, depending on the vehicle and the concentration, can be either suspended or dissolved. Parenteral solutions are normally prepared by dissolving the  
10 compound in a vehicle and filter sterilising before filling into a suitable vial or ampoule and sealing. Advantageously, adjuvants such as a local anaesthetic, preservatives and buffering agents are also dissolved in the vehicle. To enhance the stability, the composition can be frozen after filling into the vial and the water removed under vacuum. Parenteral suspensions are prepared in substantially the same manner  
15 except that the compound is suspended in the vehicle instead of being dissolved and sterilised by exposure to ethylene oxide before suspending in the sterile vehicle. Advantageously, a surfactant or wetting agent is included in the composition to facilitate uniform distribution of the active compound. Where appropriate, small amounts of bronchodilators for example sympathomimetic amines such as  
20 isoprenaline, isoetharine, salbutamol, phenylephrine and ephedrine; xanthine derivatives such as theophylline and aminophylline and corticosteroids such as prednisolone and adrenal stimulants such as ACTH may be included. As is common practice, the compositions will usually be accompanied by written or printed directions for use in the medical treatment concerned.

25 Another embodiment involves the differentiation of the obtained ES cell lines towards neurons and in particular towards post-mitotic neurons.

In another embodiment the double mutant presenilin ES cell line can be used to differentiate into many cell lineages, including heart muscle cells, blood islands, pigmented cells, macrophages, epithelia, and fat-producing adipocytes.

30 In another embodiment the double mutant presenilin ES cell line can be used to clarify the role presenilins play in the unfolded protein response (UPR-response).

In another embodiment the double mutant presenilin ES cell line can be transformed with a specific presenilin mutant being a pathogenic presenilin Alzheimer's disease causing gene. The resulting transformed mutant ES cell line with the specific presenilin



detect said proteolytic processing of said chimeric transcription factor. When intramembrane cleavage occurs said chimeric transcription factor is not kept sequestered anymore in the membrane and can shuttle to the nucleus where it activates the expression of a reporter gene. Said reporter gene is kept under control of an inducible promoter of which activity is dependent on the release of the specific, membrane sequestered transcription factor.

In a further embodiment the chimeric molecule that is used in the reporter system comprises a fusion between the intracellular domain of Notch and the transmembrane domain of APP. Said reporter system can be used to detect intramembrane proteolytic processing by  $\gamma$ -secretase. The transmembrane domain of APP comprises said  $\gamma$ -secretase cleavage site.

In yet another embodiment the chimeric molecule of the reporter system comprises a fusion between the intracellular domain of Notch and the ecto- and transmembrane domains of APP and is set forth by SEQ ID NO: 13. In SEQ ID NO: 13 APP starts at nucleotide 275 and ends at 2266. The transmembrane domain of APP starts at 2147 and ends at 2208. The intracellular domain of Notch starts at 2273 and ends at 4249. The myc tag starts at 4256 and ends at 4285. The stop codon of the chimeric protein is between 4286-4288.

In yet another embodiment the reporter system of the present invention can be used to screen for modulators and/or proteases for intramembrane proteolytic processing. In a particular embodiment the genetic background that is used to screen for said modulators and/or proteases is devoid of the activity of intramembrane proteolytic processing. In such a genetic background there is no background activation of the reporter construct since the chimeric transcription factor is maintained in the membrane. For example, the double presenilin ES cell line of the present invention is a perfect tool for the isolation of the  $\gamma$ -secretase or modulators of  $\gamma$ -secretase activity. By 'isolation' it is preferentially meant 'screening', and more preferentially 'selection'. As has been said before  $\gamma$ -secretase is an enzyme involved in Rip of the transmembrane domain of APP. In order to establish a successful cloning experiment the mutant ES cell line is first adapted into a suitable reporter ES cell line with the introduction, by transfection, of the above described reporter system. Screening for  $\gamma$ -secretase (a protease) or modulators of  $\gamma$ -secretase activity can be carried out with a suitable reporter gene, such as the green fluorescent protein. However, one can also select for  $\gamma$ -secretase or modulators of  $\gamma$ -secretase activity by use of a selection marker, such as



substrate for proteolytic processing and the second construct is made by splicing of a genetic element that is responsive to said transcription factor to a reporter gene.

**The following paragraphs clarify some terms used above and in the claims:**

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The 'ES cell' is preferably a culture cell established from the inner cell mass of a murine embryo, usually isolated at the age of 3.5 days. 'Compound' means any chemical or biological compound, including simple or complex inorganic or organic molecules, peptides, peptido-mimetics, proteins, antibodies, carbohydrates, nucleic acids or derivatives thereof. 'Wild type' is an animal (e.g. a mouse) or cell line that is isogenic with the mutant animal (e.g. mouse) or cell line except for the mutation or mutations induced in said mutant. 'Gene' means a functional promoter sequence fused to a sequence that can be transcribed, due to the activity of said promoter, into mRNA, and subsequently translated into protein, eventually after processing the mRNA by a process such as mRNA splicing. Said promoter may be the endogenous promoter of the transcribed sequence, or a heterologous promoter. 'Mutant (ES) cell line' is a (ES) cell line genetically modified by a procedure known to the people skilled in the art such as random mutagenesis, retroviral or adenoviral or lentiviral insertion, transposon mutagenesis, heterologous or homologous recombination. 'A mutant gene' is a variant of the wild type gene that differs from the wild type gene by a change, insertion and/or deletion of at least one amino acid. 'Transgenic mouse' is a mouse derived from the mutant ES cell line. It also includes an individual animal in all stages of development, including embryonic and fetal stages. A 'transgenic animal' is an animal containing one or more cells bearing genetic information received, directly or indirectly, by deliberate genetic manipulation at a subcellular level, such as by microinjection or infection with recombinant virus. This introduced DNA molecule may be integrated within a chromosome, or it may be extra-chromosomally replicating DNA. The term 'germ cell-line transgenic animal' refers to a transgenic animal in which the genetic information was introduced into a germ line cell, thereby conferring the ability to transfer the information to offspring. If such offspring in fact possess some or all of that information, then they too are transgenic animals. The information may be foreign to the species of animal to which the recipient belongs, foreign only to the particular individual recipient, or genetic information already possessed by the recipient. In the last case, the introduced gene may be differently expressed compared to the native endogenous

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heterozygous intercrosses could not be detected. At E9.5 homozygous PS1<sup>-/-</sup>PS2<sup>-/-</sup> embryos could be recovered in a nearly Mendelian distribution (15/66), but embryos were developmentally retarded by approximately half a day when compared to heterozygous littermates. Vasculogenesis of the yolk sac was delayed in most of the mutants. Although an initial vascular plexus and primitive red blood cells had formed, organisation into a discrete network of vitelline vessels was always lacking. Furthermore, yolk sacs did not expand properly and often had a blistered appearance. The embryo itself was always devoid of blood circulation and appeared posteriorly truncated. Heart development was largely unaffected, with the exception of an occasional enlarged pericardial sac. Somitogenesis had begun and turning occurred in the majority of the mutants. The optic and otic vesicle, the first branchial arch and the forelimb buds were visible. Mutants had a vestigial fore- and hindbrain, and fusion of headfolds was delayed. The neural tube had often a kinked appearance, which may be secondary to the circulation problems. This phenotype of the double deficient embryos is clearly different from that of PS2<sup>-/-</sup> embryos which appear normal and PS1<sup>-/-</sup> embryos which are only marginally retarded at E9.

## 2. Generation of double presenilin deficient ES cells

It is well known in the art that cells from the inner cell mass of mammalian blastocysts can be maintained in tissue culture under conditions where they can be propagated indefinitely as pluripotent embryonic stem (ES) cells (Thomson *et al.*, 1998, Science 282, 1145). As such blastocysts from double presenilin knockout mice were rescued from 2 days old embryos and the mutant embryonic stem cells were generated by cultivation *in vitro* according to the method of US 6103523. In Figure 1 a Southern blot is shown demonstrating the presence of wild type or knockout alleles of presenilin 1 in ES cell lines obtained from blastocysts generated by mating PS<sup>+/-</sup>PS2<sup>-/-</sup> mice.

## 3. Determination of $\gamma$ -secretase activity

In figures 2 and 3 the analysis of APP processing, containing the Swedish clinical mutation, in a double presenilin knockout background is presented. It is shown that no amyloid peptide production is observed in the double presenilin deficient ES cells.

The total gamma-secretase activity in cells is measured by assessing the release of the amyloid peptide in the culture medium from cells transfected with cDNA coding for either wt APP or APP containing the Swedish type of mutation. Measurement is done



## 5. Development of a reporter system for gamma-secretase and use of said system in cell lines.

The principle of the assay is depicted in figure 4A. An APP/Notch chimeric protein is generated. This protein contains the APP ecto- and transmembrane domains, fused to the Notch intracellular domain. The Notch intracellular domain (NIC), when cleaved, translocates to the nucleus and activates a reporter gene construct containing a defined part of the Hes 1 promotor controlling Luciferase expression. Thus proteolytic cleavage of the chimeric protein is directly linked to luciferase activity. The chimeric protein and the luciferase reporter are transiently or stably transfected in Hela-cells, HEK293 cells, COS-cells, Embryonic stem cells and other. The chimeric protein is cleaved by  $\alpha$ -secretase,  $\beta$ -secretase and  $\gamma$ -secretase. The  $\gamma$ -secretase cleavage of the construct is dependent on PS expression. In ES cells lacking PS1 and PS2 transfected with the luciferase reporter and the chimeric protein and a control plasmid, no significant Luciferase activity is induced. If an expression plasmid coding for PS1 is cotransfected however, luciferase activity is induced. Transfection experiments using the intracellular domain of the chimeric protein alone (bypassing the need for  $\gamma$ -cleavage) results in much stronger activity of the luciferase reporter in Hela cells than obtained with the APP/Notch chimeric. Therefore both decreased and increased  $\gamma$ -secretase cleavage can be assessed with this assay.

This assay can be used as a screening assay for compounds that inhibit or stimulate  $\gamma$ -secretase activity. In such assays compounds are added to cells expressing the chimeric and reporter protein. After a defined period of time (24 or 48 hours, but shorter periods of time can be chosen), luciferase activity is measured in the treated cells and in the control cells. Changes in luciferase activity are an indicator of decreased or increased  $\gamma$ -secretase activity. Compounds that selectively decrease luciferase activity in cells expressing the chimeric protein and reporter but not in cells transfected with the intracellular part of the chimeric protein alone are not toxic to the cells and are likely specific inhibitors for the  $\gamma$ -secretase.

This assay can also be used to screen for cDNA's coding for proteins that modulate  $\gamma$ -secretase activity. In this type of experiments cDNA's from a cDNA library either using classical transfection protocols or using viral transduction (adeno-, Semliki Forest- or other viral vectors) are transfected into cells or cell lines expressing transiently or stable the chimeric protein and the reporter. Positive hits (significant up- or down regulation of luciferase activity) can be selected from these screens. The



## 6. Development of a cell free assay

The mutant ES cell line can be used as the basis for a cell free assay following the generation and release of transport vesicles from endoplasmic reticulum. The same type of studies has been critical for the unravelling of the molecular sorting machinery in eukaryotic cells. One can isolate ER-fractions from wild type and PS1-/-mice and the export budding from the ER can be reconstituted using small GTP binding proteins and recombinant COPII coat proteins. Newly formed vesicles can be isolated and analyzed for amyloid peptide generation. The molecular composition of the vesicles can be analyzed using 2D gel electrophoresis combined with amino acid sequencing. Cross-linking experiments allow to identify components of the postulated presenilin-APP- $\gamma$ -secretase complex. By comparing protein profiles from isolated vesicles generated from material from the double deficient cell line, with that from wild type PS expressing ES cells, it is possible to identify proteins that are differentially present in the vesicles. Analyses by two-dimensional PAGE and amino acid sequencing of differentially detected protein spots will yield new proteins whose processing and transport is regulated by presenilins. Furthermore it is envisaged that proteins like  $\gamma$ -secretase or other proteases that are dependend on the presence of presenilins will be either decreased or increased in these samples. Further amino acid sequencing of these spots will yield  $\gamma$ -secretase and other protease candidates involved in RIP (regulated intramembrane proteolysis) (Brown *et al.*, 2000, Cell 100, 391).

## 7. Inhibitors of $\beta$ -amyloid<sub>42</sub> peptide production

In another example the mutant ES cell line is transformed with the wild type PS1 gene resulting in transformant 1 while transformant 2 is the mutant ES cell line genetically transformed with a specific familial Alzheimer's disease causing mutation in PS1 or PS2 or combination of mutations. It is expected from the state of the art that transformant 2 predominantly produces the amyloid- $\beta$ -42 form whereas transformant 1 mostly produces the non-pathogenic amyloid- $\beta$ -40 peptide. These two transformants can be used in a differential drug screening approach to identify compounds which inhibit preferentially the amyloid- $\beta$ -42 formation and not the amyloid- $\beta$ -40 peptide generation. In a first screening with transformant 2 compounds are identified which specifically inhibit the formation of the amyloid- $\beta$ -42 peptide. Specific monoclonal antibodies exist which can differentiate between the amyloid- $\beta$ -42- and amyloid- $\beta$ -40



APP-NIC (fig 4A and 4B) fusion constructs, also called chimeric proteins, were made using the human APP695 sequence and the mouse Notch-1 sequence. The APP encoding sequence (residues -20bp to +1992bp, the ATG of the APP open reading frame being number 1) was constructed by joining the SmaI-SacI fragment encoding bases (-20bp to +1692) to a PCR fragment encoding bases (+1693bp to +1992bp) generating a unique EcorV site and a Myc tag (EQKLISEEDL) at the 3' end. Oligonucleotides used were: 5'-AACCACCGTGGAGCTCCTTC-3' and 5'-CCAAGCTTCTACAAGTCCTCTTCAGAAATCAGCTTTTGCTCGTTAACGATATCGTCAACCTCCACCACACCATG-3'. Three different cDNA fragments encoding part of the Notch-1 intracellular domain (+5286bp to +6291bp, +5286bp to 7251bp, 5286bp to 7554bp respectively, bp 1 being the ATG) were generated by PCR on a mouse brain cDNA library and subcloned into the EcoRV site giving rise to APP:NIC2, APP:NIC, and APP:NIC1 (see fig4B). Oligonucleotides used were: 5'-CACCCGGGTTCCCTGAGGGTTTCAAAGT-3', 5'-CCGCACGATATCGTGGTG-3', 5'-GCGTTAACATCTGCCTGACTGGGCTC-3' and 5'-CAGTTAACGGTGGTGGGCGGGCTGGAGAT-3'. A SV40 polyadenylation signal (isolated from pSG5, Stratagene) was added as indicated in the figure 4B. The mouse Pgk-1 promotor (-456bp to -18bp, Gene, 60, 65-74, 1987) or the SV40 early promotor from pSG5 (Stratagene) was cloned in the unique SmaI site of the fusion constructs.

The K595N;M596L (numbering as in APP 695, the M encoded by ATG is 1) Swedish mutation was introduced into the pSG5APP:NIC plasmid by site directed mutagenesis (Stratagene) using the following primer 5'-GGAGATCTCTGAAGTGAATCTGGATGCAGAATTCCGAC -3'. This construct was called pSG5APPSw:NIC. pSG5 $\beta$ A4:NIC was generated by replacing the APP ectodomain of pSG5APP:NIC with the APPC99 stub. APPC99 contains the carboxyterminal 99 amino acid residues of the APP sequence (thus starts with the  $\beta$ -cleavage site in APP). To obtain the correct cleavage by signal peptidase in the APPC99 stub an extra DA motif was added between the signal sequence and the  $\beta$ -cleavage site. A NIC construct containing only the intracellular part of the chimeric protein was generated by PCR with the following oligonucleotides 5'-AGGATCCATGGTGCTGCTGTCCCGCAAGCGCCGGCGGCAGCATGGCCAGCTCTGGTTCCCTGAGGGTTTCAAAGTGT-3' and 5'-GCGTTAACATCTGCCTGACTGGGCTC-3. The fusion APP:NIC and the NIC construct were cloned in pCDNAzeo (invitrogen) to generate stable cell lines.



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Original (for SUBMISSION) - printed on 21.02.2001 11:49:32 AM

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1-3-1	Name of depositary institution	Vakgroep voor Moleculaire Biologie - Plasmidencollectie (BCCM/LMBP)
1-3-2	Address of depositary institution	Universiteit Gent, K.L. Ledeganckstraat 35, B-9000 Gent, Belgium
1-3-3	Date of deposit	24 February 2000 (24.02.2000)
1-3-4	Accession Number	LMBP 5472CB
1-4	Additional Indications	NONE
1-5	Designated States for Which Indications are Made	all designated States
1-6	Separate Furnishing of Indications These indications will be submitted to the International Bureau later	NONE

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0-5-1	Authorized officer	



- said  $\gamma$ -secretase activity in said embryonic stem cell line is monitored.

10. Use of an embryonic stem cell line according to any of claims 1-7 for identifying a compound which specifically interferes with the formation of the A $\beta$ 42-peptide and not with the formation of the A $\beta$ -40 peptide characterized in that:

- said embryonic stem cell line is transfected with at least one mutated gene coding for presenilin 1 and/or presenilin 2 and/or amyloid  $\beta$  precursor protein, and
- said transfected stem cell line is exposed to at least one compound whose ability to interfere with the formation of the A $\beta$ -42 peptide and not with the formation of the A $\beta$ -40 peptide is sought to be determined, and
- said formation of A $\beta$ -42 peptide is monitored.

11. A method for the production of a pharmaceutical composition comprising the usage of an embryonic stem cell line according to claim 9 or 10 and further more mixing the gene or compound identified or a derivative or homologue thereof with a pharmaceutically acceptable carrier.

12. A transgenic mouse obtained by using an embryonic stem cell line according to any of claim 1 to 4 comprising:

- transfecting said mutant ES cell line with a pathogenic presenillin Alzheimer disease causing gene and,
- injecting the resulting transfected mutant ES cell line into a blastocyst and,
- implanting said injected blastocyst into a female mouse.

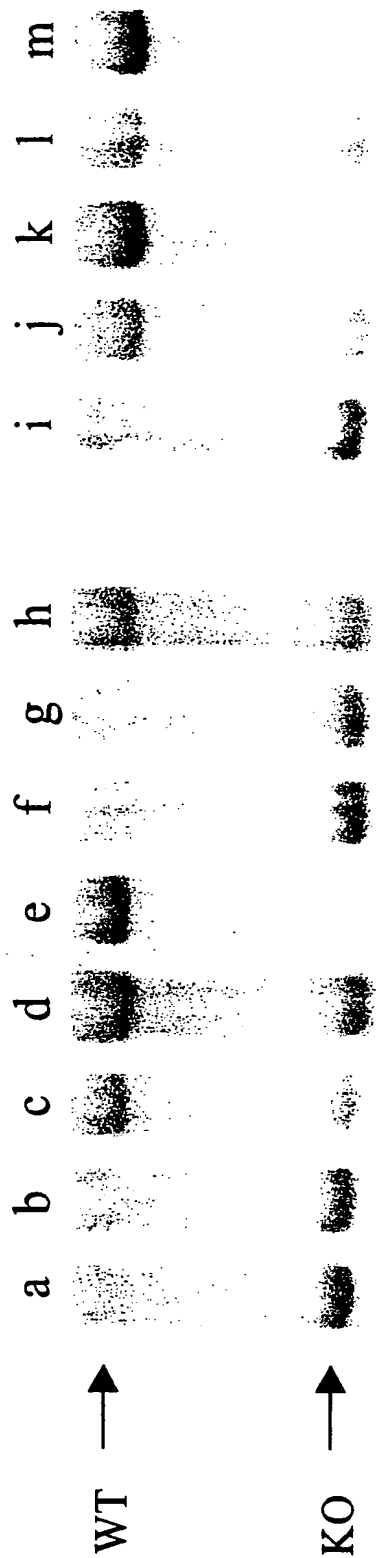
13. A reporter system to detect intramembrane proteolytic processing comprising:

- a chimeric molecule comprising a fusion between a transcription factor and a transmembrane domain that is known to be a substrate for proteolytic processing, and
- a reporter construct that detects said proteolytic processing of said chimeric transcription factor.

14. A reporter system according to claim 13 wherein said chimeric molecule comprises a fusion between the intracellular domain of Notch and the transmembrane domain of APP.



Figure 1



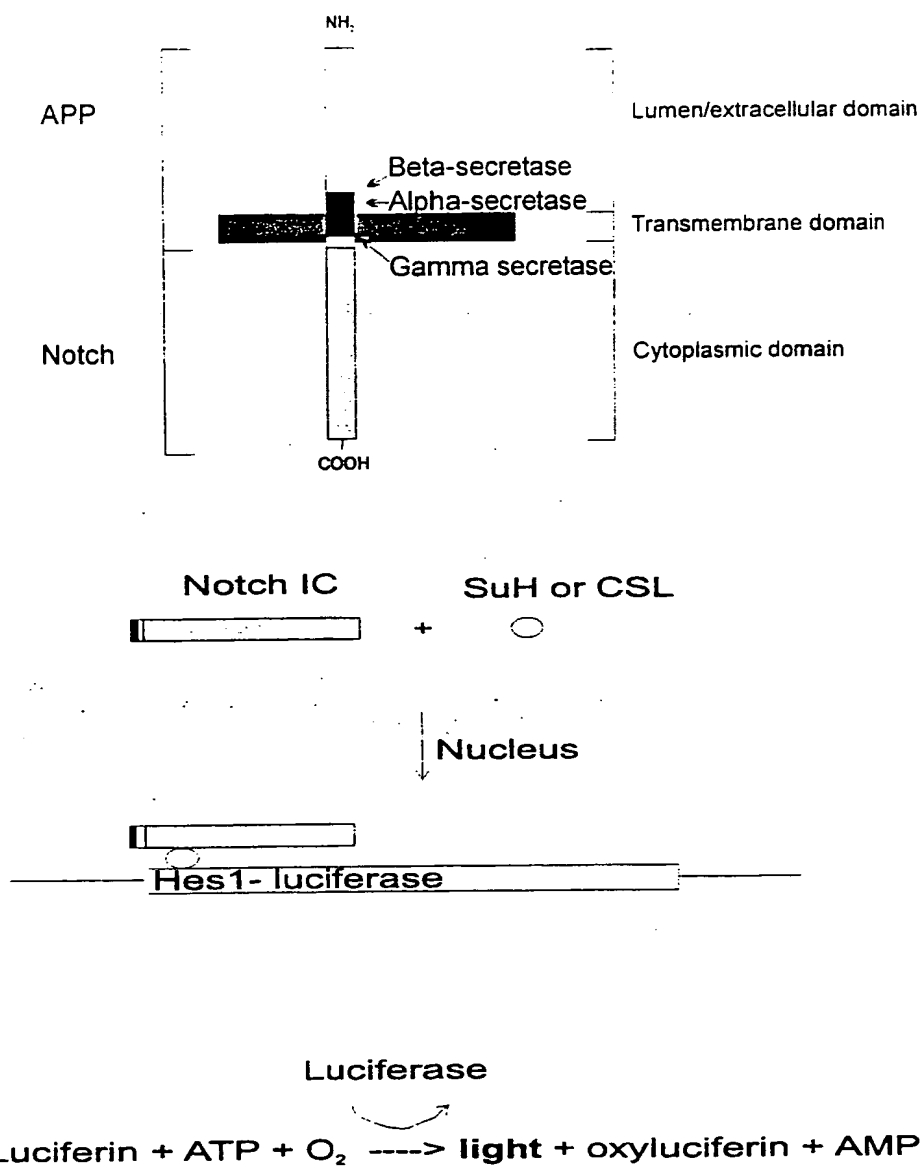


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Figure 4 A

## Principle

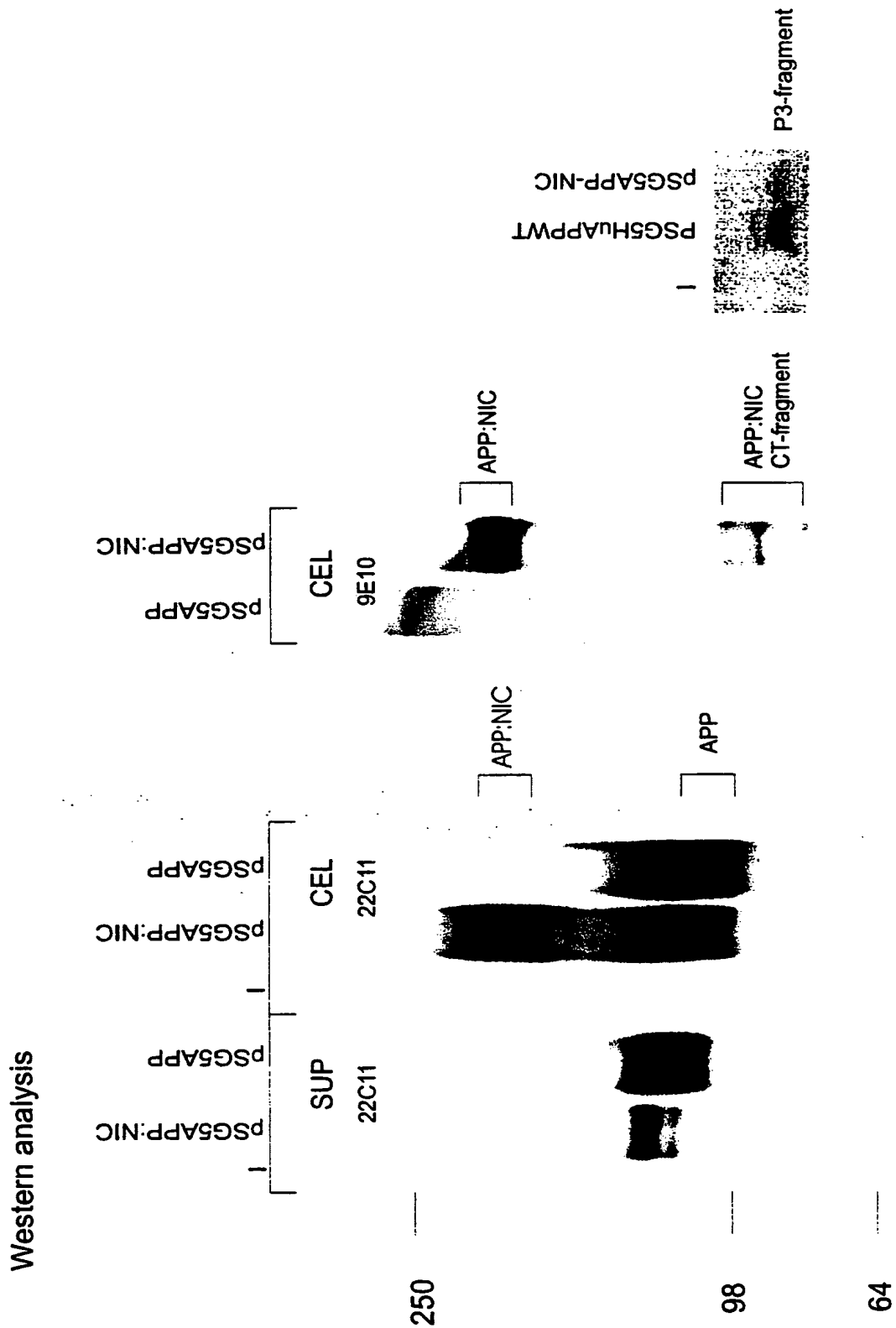
## Chimeric APP : NIC construct





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Figure 4 C





## SEQUENCE LISTING

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<120> Presenilin deficient multipotent cell lines and  
screening methods for intramembrane regulated  
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## INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 01/02127

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>HERREMAN AN ET AL: "Presenilin 2 deficiency causes a mild pulmonary phenotype and no changes in amyloid precursor protein processing but enhances the embryonic lethal phenotype of presenilin 1 deficiency."  PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES, vol. 96, no. 21, 12 October 1999 (1999-10-12), pages 11872-11877, XP002173228  Oct. 12, 1999  ISSN: 0027-8424  cited in the application abstract  page 11875, right-hand column, last paragraph -page 11877, left-hand column, paragraph 1</p>	1,2,8,12
A	<p>DE STROOPER BART ET AL: "A presenilin-1-dependent gamma-secretase-like protease mediates release of Notch intracellular domain."  NATURE (LONDON), vol. 398, no. 6727, 8 April 1999 (1999-04-08), pages 518-522, XP001010555  ISSN: 0028-0836  the whole document</p>	1,10,13
A	<p>KIMBERLY W TAYLOR ET AL: "The transmembrane aspartates in presenilin 1 and 2 are obligatory for gamma-secretase activity and amyloid beta-protein generation."  JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 275, no. 5, 4 February 2000 (2000-02-04), pages 3173-3178, XP002173229  ISSN: 0021-9258  the whole document</p>	1-4,10
A	<p>WOLFE MICHAEL S ET AL: "Two transmembrane aspartates in presenilin-1 required for presenilin endoproteolysis and gamma-secretase activity."  NATURE (LONDON), vol. 398, no. 6727, 8 April 1999 (1999-04-08), pages 513-517, XP001010554  ISSN: 0028-0836  the whole document</p>	1-4

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International Application No

PCT/EP 01/02127

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO 00 34511 A (AVENTIS PHARMA GMBH) 15 June 2000 (2000-06-15) abstract page 3, line 4 -page 6, line 6 examples 1-3 -----	1,3,4, 9-11,13
P,X	WO 00 68416 A (BOEHRINGER INGELHEIM PHARMA ;HAASS CHRISTIAN (DE); PESOLD BRIGITTE) 16 November 2000 (2000-11-16) abstract page 3, line 20-35 page 6, line 28 -page 8, line 28; figures 4,5; examples 1,2 -----	13
P,X	HERREMAN AN ET AL: "Total inactivation of gamma-secretase activity in presenilin-deficient embryonic stem cells." NATURE CELL BIOLOGY, vol. 2, no. 7, July 2000 (2000-07), pages 461-462, XP001015216 ISSN: 1465-7392 the whole document -----	1-18
P,X	ZHANG ZHUOHUA ET AL: "Presenilins are required for gamma-secretase cleavage of beta-APP and transmembrane cleavage of Notch-1." NATURE CELL BIOLOGY, vol. 2, no. 7, July 2000 (2000-07), pages 463-465, XP001015214 ISSN: 1465-7392 the whole document -----	1,10,12
P,X	ZHANG J ET AL: "APP chimeric proteins containing the Notch trans-membrane domain are cleaved by gamma-secretase." SOCIETY FOR NEUROSCIENCE ABSTRACTS, vol. 26, no. 1-2, 2000, pages Abstract No.-180.12, XP001014404 30th Annual Meeting of the Society of Neuroscience; New Orleans, LA, USA; November 04-09, 2000 ISSN: 0190-5295 abstract -----	13-18

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